°FORM PTO-1390 (REV 11-2000) U.S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
491332000300	10/018842 to be assigned

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/BR00/00068	June21, 2000	June 22, 1999

TITLE OF INVENTION

SYNTHESIS OFA NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELLING CHEMICAL AND BIOLOGICAL MACROMOLECULES

APPLICANT(S) FOR DO/EO/US

Clovis Ryuichi NAKAIE et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information: a copy of the published international application; FORM PCT/IB/308; FORM PCT/IPEA/403; FORM PCT/IPEA/402; FORM PCT/IB/332 and return receipt postcard.

CERTIFICATE OF HAND DELIVERY

I hereby certify that this correspondence is being hand filed with the United States Patent and Trademark Office in Washington, D.C. on December 21, 2001.

Jinrong Li

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) to be assigned 10/018842	INTERNATIONAL APPLICATION NO. PCT/BR00/00068	ATTORNEY'S DOCKET NUMBER: 491332000300

21. ☐ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1,040.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provision of PCT Article 33(1)-(4) $710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$100.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$*	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	11- 20 =	*	x $18.00	$*	
Independent claims	3 - 3 =	*	x $84.00	$*	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$*	

TOTAL OF ABOVE CALCULATIONS =	$*	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.	$*	
SUBTOTAL =	$*	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$*	
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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00 per property** +	$*	
TOTAL FEES ENCLOSED =	$100.00	
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	charged:	$*

a. ☐ A check in the amount of $* to cover the above fees is enclosed.

b. ☒ Please charge my **Deposit Account No. 03-1952** in the amount of $100.00 referencing Docket No. 491332000300 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees that may be required, or credit any overpayment to **Deposit Account No. 03-1952**. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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10/018842
JC13 Rec'd PCT/PTO 21 DEC 2001

Application Data Sheet

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Application Type:	Utility
Docket Number:	491332000300

Representative Information

Representative Customer Number:	25227

Continuity Information

This application is a:	Continuation of
> Application One:	PCT/BR00/00068
Filing Date:	June 21, 2000.

Prior Foreign Applications

Foreign Application One:	PI 9903137-0
Filing Date:	June 22, 1999
Country:	Brazil
Priority Claimed:	Yes

PATENT
Docket No. 491332000300

CERTIFICATE OF HAND DELIVERY

I hereby certify that this correspondence is being hand filed with the United States Patent and Trademark Office in Washington, D.C. on December 21, 2001.

Jinrong Li

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Clóvis Ryuichi NAKAIE et al.

Serial No.: Based on PCT/BR00/00068

Filing Date: Concurrently Herewith

For: SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELING DIFFERENT MACROMOLECULES AND SYSTEMS (as amended)

Examiner: To Be Assigned

Group Art Unit: To Be Assigned

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to the calculation of the filing fee for this application, please enter the following amendments:

AMENDMENTS

In the Specification:

Please replace the specification as filed with the substitute specification attached at the end of this Preliminary Amendment.

Please enter the attached Abstract of the Disclosure.

In the Claims:

Cancel claims 1-3 without prejudice or disclaimer and replace them with new claims 4-12, as follows:

4. 2,2,5,5-tetramethylpyrrolidine-n-oxyl-(9-fluorenylmethyloxycarbonyl)-3-amine-4-carboxylic acid.

5. Poac^{7}-Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Poac-Phe).

6. A method of labeling a molecule or system for chemical or biochemical study, comprising coupling the molecule or system with 2,2,5,5-tetramethylpyrrolidine-n-oxyl-(9-fluorenylmethyloxycarbonyl)-3-amine-4-carboxylic acid.

7. The method of claim 6, wherein the molecule or system comprises a carboxyl or amine reactive function.

8. The method of claim 6, wherein the chemical or biochemical study is electron paramagnetic resonance.

9. The method of claim 6, further comprising removing the 9-fluorenylmethyloxycarbonyl group from the 2,2,5,5-tetramethylpyrrolidine-n-oxyl-(9-fluorenylmethyloxycarbonyl)-3-amine-4-carboxylic acid to produce the molecule or system with an unprotected Poac derivative.

10. The method of claim 9, further comprising coupling a chemical group at a free amine function of the molecule or system containing the unprotected Poac derivative.

11. The method of claim 6, wherein the molecule is angiotensin-II.

12. The method of claim 6, wherein the molecule is α-melanocyte stimulating hormone.

REMARKS

Applicants have amended the specification and claims to put this application in better condition for examination in the United States. The additions to the application include a Brief Description of the Drawings, an Abstract of the Disclosure based on the abstract accompanying the published International Application and claims directed to aspects of the invention disclosed but not claimed in the International Application as filed. These amendments introduce no new matter into this application.

Early action allowing claims 4-12 is solicited.

Attached hereto is a marked-up version of the changes made to the specification by this amendment, captioned "**Version marked to show changes made**".

In the event that the transmittal letter is separated from this document and the Patent and Trademark Office determines that an extension and/or other relief is required, applicants petition for any required relief including extensions of time and authorize the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing 491332000300.

Respectfully submitted,

Dated: December 21, 2001

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ABSTRACT OF THE DISCLOSURE

The present invention refers to the synthesis and application of 2,2,5,5-tetramethylpyrrolidine-N-oxyl-(9-fluorenylmethyloxycarbonyl)-3-amine-4-carboxylic acid, a novel paramagnetic amino acid derivative (spin label), denominated Fmoc-Poac. Fmoc-Poac can be coupled to peptide sequences and other molecules or systems. It can be inserted anywhere in a peptide segment, even at an internal position if necessary, after removal of its temporary amine protecting group, Fmoc. Owing to its pyrrolidine structure, this molecule may induce differentiated conformations as compared with normal α-amino acids, thus being a valuable probe for structural-biological activity of several relevant peptides. The Poac-angiotensin II analogue was synthesized as a model according to its use as a chemical derivative.

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Please amend the specification as follows:

SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELING DIFFERENT MACROMOLECULES AND SYSTEMS ~~OF CHEMICAL-BIOLOGICAL INTEREST~~

FIELD OF THE INVENTION

This invention ~~refers~~ **relates** to the chemical synthesis of a new paramagnetic β-amino acid derivative containing ~~the~~ **a** stable nitroxide radical moiety inserted in its pyrrolidine structure and **in which** the 9-fluorenylmethyloxycarbonyl group (Fmoc) was chosen as its amine function protecting group. This paramagnetic compound is ~~therefore~~ a new type of spin probe (or spin label) and ~~it~~ can be used as alternative report molecule for labeling peptide sequences, other macromolecules and systems where ~~the~~ electron paramagnetic resonance spectroscopy (EPR) can be applied. ~~The use of this~~ **This** compound can be ~~extended~~ **used** also for other spectroscopic methods such as fluorescence and nuclear magnetic resonance since its paramagnetism may affect the spectra of ~~this~~ **these** methodologies. Due to the presence of both carboxyl and amine groups in its structure, ~~the use of~~ this organic compound may be ~~extended~~ **used** for labeling a great variety of other molecules or systems containing reactive ~~function~~ **functions** for these two groups.

BACKGROUND AND SUMMARY OF THE INVENTION

The intermediate for the synthesis of the amino acid derivative of ~~the present patent~~ **this invention** contains the structure 2,2,5,5-tetramethylpyrrolidine-1-~~oxil~~ **oxyl**-3-amine-4-carboxylic acid**,** henceforth denominated as Poac, synthesized more than two decades ago ~~(v.g.~~**(see,** Tetrahedron 491-499 [1965] and Bull. France, 3, 815-817 [1967]). ~~Thus, the~~ **The** Poac derivative containing the Fmoc protecting group (2,2,5,5-tetramethylpyrrolidine-1-~~oxil-3-(9-~~

~~fluorenylmethyloxicarbonyl)~~ **oxyl-3-(9-fluorenylmethyloxycarbonyl)**-amine-4-carboxylic acid) is the novel spin probe derivative of the present ~~patent of invention~~ **invention, which also includes the synthesis and use in EPR of this spon probe derivative**. This new compound allows the ~~POAC~~ insertion **of Poac** as ~~an~~ **a** usual amino acid at any position of ~~the~~ **a** peptide sequence, and its denomination will be Fmoc-Poac or EPM-5 in this ~~descriptive report~~ **application**. The chemical structure of this paramagnetic molecule is represented in Figure 1.

~~The electron~~ **Electron** paramagnetic resonance (EPR) [**described** in *Biological Magnetic Resonance*, Berliner, L. J. and Reuben, J., eds., Plenum Publishing, New York, (1989)], is a modern and very useful spectroscopic method because it allows ~~studies~~ **the study** of ~~any~~ paramagnetically labeled macromolecules or biological systems regarding their conformation, mobilities, inter- or intra-molecular interactions, structuring ~~state, etc~~ **states, and the like**. The wide spectrum of EPR application is already detailed in the literature ~~(v.g.~~**(see,** *Free Nitroxyl Radical*, Rozantsev, E.G., Ulrich, H., ed., Plenun Press, London, 1970), where a great variety of spin labels, i.e., chemical compounds which are paramagnetic due to the presence of an unpaired electron in ~~its~~ **their** structure, is listed. They are, therefore, a class of free ~~radical~~ **radicals** but ~~must be~~ **are** necessarily stable ~~towards~~ **under conditions around** normal temperature and physiological pH and also allow several chemical reactions or experiments without affecting ~~its~~ **their** free radical moiety.

Amongst the most commonly used spin labels one can ~~detach~~ **pick out** the nitroxide group-containing molecules and where the unpaired electron locates. The most significant progress in the ~~RPE~~ **EPR** field for labeling of relevant biological structures such as peptides and proteins was achieved with this class of spin probes. Almost two decades ago appeared the first ~~RPE~~ **EPR** application in the solid phase peptide synthesis methodology [(*The Peptides: Analysis, Synthesis and Biology*, vol. 2, Academic Press, New York, (1980)]. This approach was introduced by our group using, instead, ~~an other~~ **another** nitroxide-containing spin label,

denominated at that time as Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine-4-carboxylic acid**), which** protected ~~at it~~ **its** amine function with the acid labile tert-~~butiloxicarbonyl~~ **butyloxycarbonyl** (Boc) group [~~(v.g.~~**(see,** Braz. J. Med. Biol. Res. *14*, 173, (1981) and Biochim. Biophys. Acta, *742*, 63, (1983)]. Thus the Boc-Toac spin probe was the first in the literature used to label a peptide sequence as an amino acid. However, due to chemical particularities of the peptide synthesis methodology, it was only possible to couple the Toac group at the peptide N-terminal position. To overcome this shortcoming, an alternative strategy was published by us which finally allowed the insertion of the spin label internally to the peptide sequence [~~v.g.~~ **see,** J. Am Chem. ~~Soc.115~~ **Soc. *115***, 11042 (1993)].

An impressive increase in the application of ~~RPE~~ **EPR** for peptide chemistry field was further observed ~~with~~ **in** reports investigating peptide conformational properties [v.g. J. Am. Chem. Soc. 117, 10555 (1995); FEBS Lett. *375*, 239 (1995); Biopolymers *42*, 821 (1997)] or of ~~peptidil~~ **peptidyl**-resin solvation (Tetrahedron Lett. *375*, 239 [1995]; Biopolymers *42*, 821 [1997]). As an amino acid, ~~TOAC~~ **Toac** was introduced in different positions of some biologically active peptides such as angiotensin II and bradykinin**,** but a partial or ~~integral~~ **total** loss of their biological potencies were observed due to the introduction of a non natural compound in their structures. [*Peptides 1996*, R. Ramage and R. Epton, eds**.**, Mayflower Scientific Co. p. 673~~,~~ (1998)].

However, we recently described the synthesis of a peptide hormone labeled with Toac ~~and~~ where its biological potency was entirely preserved. [~~v.g~~ **e.g.,** FEBS Lett. *446*, 45 (1999)]. This result was obtained with the tridecapeptide α-melanocyte stimulating hormone analogue ~~and~~**,** owing to its potentialities in a great number of chemical-biological assays (this analogue is paramagnetic, naturally fluorescent and fully active~~), was submitted for the patent process (PI 9900595, February 24, 1999 : "Synthesis of the first paramagnetic and active -melanocyte stimulating hormone analogue containing free radical amino acid"]~~**., and was disclosed in**

commonly owned U.S. patent application Serial No. 09/935, 760, entitled "Paramagnetic And Active Analogue (EPM-2) Of Melanocyte Stimulating Hormone Containing Amino Acid-Type Stable Free Radical").

In spite of these results, one still remaining shortcoming ~~to~~ **in** the use of Toac in peptide chemistry ~~refers to~~ **is** the severe difficulty in coupling the subsequent amino acid residue of the peptide sequence during the synthesis. ~~It~~ **This difficulty** seems to be due to the low nucleophilicity of **the** Toac amine group, whose pKa of 8 (when in free state) decreases to about 5.5 when bound to the N-terminal portion of a peptide chain [v.g., Braz. J. Med. Biol. Res. *14*, 173 (1981) and Biochim. Biophys. Acta. *742*, 63 (1983)]. Several recouplings and ~~the~~ **an** increase ~~on~~ **in** the temperature of the coupling reaction are usually necessary to assure complete incorporation of the subsequent amino acid of the desired peptide sequence [J. Am. Chem. ~~Soc.115, 11042 (1993)].~~ **Soc. *115*, 11042 (1993)].**

~~**Aiming to overcome this limitation inherent to the Toac probe and searching for**~~ **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a depiction of the structure of Fmoc-Poac or EPM-5 according to this invention.

Figure 2 is a mass spectrum of Poac.

Figure 3 is a high performance liquid chromatography (HPLC) profile of Poac showing a single peak.

Figures 4A and 4B depict EPR spectra of Poac in water and Fmoc-Poac in dimethylformamide, respectively.

Figure 5 is a mass spectrum of Fmoc-Poac.

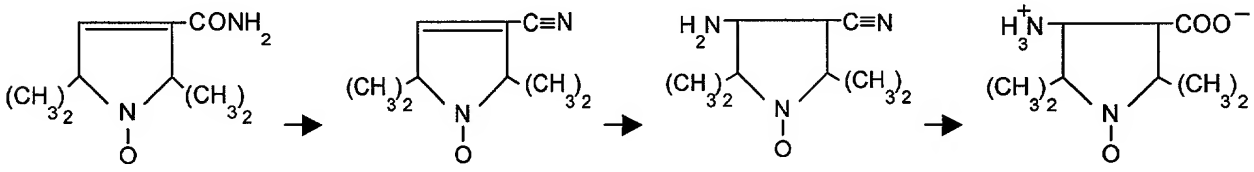
Figure 6 is a HPLC profile of Poac^7 showing a single peak.

Figure 7 is a mass spectrum of Poac^7-angiotensin II.

Figures 8A, 8B and 8C depict EPR spectra of Poac[7]-angiotensin II at pH 3.0, 6.0 and 9.0, respectively.

DETAILED DESCRIPTION OF THE INVENTION

In order to overcome the limitations of the Toac probe to find an alternative spin label which may induce ~~a~~ differentiated conformational constraints in peptide structures~~, we decided to synthesize the FMOC-POAC according to partially described~~ **in accordance with this invention, we synthesized Fmoc-Poac according to the** synthetic route shown below ~~[~~**that is partially described in** Tetrahedron, 491-499 (1965); Bull. Soc..Chim. France, *3*, 815 (1967)~~]~~):



P1 **P2** **P3** **P4**

Step #1 - Synthesis of 2,2,5,5-~~tetrametilpyrroline-1-oxil~~ **tetramethylpyrroline-1-oxyl**-3-cyano (P2)

This product was synthesized by treating ~~the~~ compound P1 (from Sigma Co) with ~~tosil~~-**tosyl (p-toluenesulfonate)** chloride in dry pyridine. To 28.7 g (1.5×10^{-1} mol) of ~~tosil~~-**tosyl** chloride, 15.3 g (8.35×10^{-2} mol) of P1 dissolved in 100 mL of dry pyridine was added and left at room temperature for 48 hours. After this period, 10 g of KOH dissolved in 250 mL of water were added, and the mixture was heated up ~~for~~ **to** 80°C. After cooling, the product was extracted with sulfuric ether, washed with diluted HCl, diluted $NaHCO_3$ solution, water and dried over anhydrous Na_2SO_4. After evaporation of the solvent under reduced pressure 12.76 g (yield= 92%) of an orange powder was obtained and further purified in an alumina column using

benzene as **an** eluent. The product (P2) showed a single spot in thin layer chromatography ~~and~~ with following characteristics: M.P.= 62-63°C, M+ = 165; Elementary analysis ÷ found: C, 65.36% , H, 7.50% e N, 17.10 % ; calculated : C, 65.43% , H, 7.93% e N, 16.96%).

Step #2 : Synthesis of 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amine-4-cyano (P3).

In a 3 L round bottom flask, 600 mL of liquid ammonia, 9 g (5.44 x 10^{-2} mol) of P2 and 120 mL of water were added. The flask was tightly closed to maintain the mixture under pressure and left at room temperature. After 3 days, the ammonia was eliminated and the product extracted with chloroform. The crude product crystallized from ether-petroleum ether yielded 9.37 g (yield = 94%) of a yellow powder with the following characteristics: M+= 182; P.F. 84-85 °C; Elementary analysis ÷ found: C 58.98%; H, 8.37%; N, 22.21%; calculated ($C_9H_{16}ON_3$): C, 59. 31%; H, 8.85 % ; N, 23.06 %).

Step 3 - Synthesis of 2,2,5,5-~~tetrametilpyrrolidine-1-oxil~~ **tetramethylpyrrolidine-1-oxyl**-3-amino-4-carboxylic-acid (P4) ~~-POAC~~**(Poac).**

8 g (4.38 x 10^{-2} mol) of P3 and 40 g of $Ba(OH)_2$ were suspended in 600 mL of water and added to a 3 L round bottom flask. The flask was tightly closed and heated up to 120°C for 90 minutes. After cooling, the mixture was neutralized with **an** excess of dry ice and filtered. The aqueous solution was concentrated under reduced pressure and yielded 8 g of crude product (yield: 90%) that was crystallized from 90% ethanol. The product presented the following characteristics: M.P. = 212°C (melts with sublimation); M+ = 201 (Figure 2), single ~~pick~~ **peak** in HPLC, (Figure 3); elementary analysis ÷ found : C, 53.1% ; H, 8.28 %; N, 13.95 % ; calculated ($C_9H_{17}N_2O_3$): C, 53.71 % ; H, 8.52; N, 13.92 Infra-red (KBr) : cm^{-1} : 3084, 2872, 2792, 2548 and 2132 (NH+3); 1643 ν_{AS} (NH+3) ; 1574 (ν_{AS} , C=O); 1456 (δ CH3); 1396 and 1376 (gem-dimethyl and COO-); 782 (δ C=O). ~~The~~ Figure 4A shows the EPR spectrum of POAC in aqueous solution, pH 5. The calculated values for the two rotational correlation times (τ_B and τ_c) were 0.509 x 10^{-10} and 0.597 x 10^{-10}, respectively.

Preparation of Fmoc-Poac

201 mg (1 mmol) of P4 was dissolved in 1.5 mL of water in presence of 286 mg of sodium ~~carbonate.10 H2O~~ **carbonate.10H_2O** in which 337 mg ~~(1mmol)~~**(1 mmol)** of Fmoc-succinimidyl-carbonate dissolved in 1.5 mL of acetone was added drop-wise. The reaction was carried out at room temperature with stirring, and the pH was maintained around 9 by addition of sodium carbonate. After 3 hours, the mixture was diluted with 25 mL of water~~,~~ **and** acidified with 1 N HCl until **it reached** pH 2. The desired product was extracted with ethyl acetate, washed with small portions of water, dried over anhydrous sodium sulfate. After filtration, the solvent was evaporated under reduced pressure. The crude product was crystallized twice with chloroform and yielded 380 mg (yield : 90%). The product was characterized by mass and infra-red spectroscopy, elementary analysis and EPR. Characteristics: M+ = 423 (Figure 5); elementary analysis ÷ found: C, 67.9 % ; H, 6.35 %; N, 6.60 ; calculated ($C_{24}H_{27}O_5N_2$): C, 68. 08% ; H, 6.28 % ; N, 6.62%; IR (KBr) cm^{-1}: 3444-3338 (broad band OH and -CONHR); ~3030 (νAr CH); 3000-2700 (νAr COOH); 1723 (R-O-C-ON- and COOH); 1543 (δ_{NH} and νCN); 1450 (δ CH_3); 1235-1150 (gem-dimethyl group). The EPR spectra of Fmoc-~~POAC~~ **Poac** in dimethylformamide is represented in Figure 4B and the calculated τ_B and τ_c values are 1.14×10^{-10} $s.rad^{-1}$ and 1.79×10^{-10} $s.\ rad^{-1}$, respectively.

Synthesis of $Poac^7$-angiotensin II

Angiotensin II analogue labeled with the spin probe ~~POAC~~ **Poac** (Asp-Arg-Val-Tyr-Ile-His-Poac-Phe) was synthesized in 0.15 mmol scale, by the solid phase method already mentioned and with alteration to provide the insertion of this marker in the middle of the peptide chain. [J. Am. Chem. Soc. *115*, 11042 (1993)]. ~~FMOC~~ **Fmoc**-Phe-Wang-resin [J. Am. Chem. Soc. *95*, 1328 (1973)] with 0.41 mmol/g substitution degree acquired commercially was used. All couplings were carried out using 2.5 fold excess for the ~~FMOC~~ **Fmoc**-amino acids and 3 fold excess for Fmoc-~~POAC~~ **Poac**. The acylating reagents for coupling were

diisopropylcarbodiimide (DIC) and 1-~~hidroxibenzotriazole in~~ **hydroxybenzotriazole in a** dichloromethane:dimethylformamide mixture (1:1, v/v) as **a** solvent. The Fmoc deprotection was performed with 20% piperidine in dimethylformamide (v/v) for ~~20min~~ **20 min**.

Interestingly, this synthesis demonstrated that the pyrrolidine structure of the Poac spin label allowed much easier incorporation of the subsequent amino acid residue than ~~the~~ **has been** observed during the Toac^7-AII synthesis. In this latter case, repetitive recoupling reactions were necessary**,** including ~~the~~ **an** increase in ~~the~~ temperature. These procedures were not necessary in the case of **the** Poac ~~derivative~~ **derivatives,** thus demonstrating that the Poac amine group reactivity is much higher than that of Toac.

After the completion of the synthesis, the peptide was cleaved from the resin in anhydrous HF containing 10% of ~~the~~ **a** p-cresol and dimethylsulfide mixture, at 0°C for 90 min. The crude peptide obtained after extraction and lyophilization (125 mg) was dissolved in 70 mL of water and the pH was raised to 10 with ammonium hydroxide and stirred for 2 h, to ~~revert~~ **reverse** the nitroxide protonation that occurs during the HF treatment. After lyophilization, the peptide was purified by preparative HPLC (high performance liquid chromatography) using a reverse phase C_{18} (25 x 250 mm) and ammonium acetate 0.02 M, pH 5 and acetonitrile 60% in water, as solvents A and B, respectively. The linear gradient applied was from 20-65% of B ~~in~~ **for** 135 min.

The homogeneity of the peptide was confirmed through analytical HPLC (Figure 6), mass spectrometry, M+ = 1132.55 (Figure 7)**,** and the amino acids analysis showed the expected composition : Asp 0.95 (1.00); Val 0.96 (1.00); Ile 1.20 (1.00); Tyr 1.02 (1.00); Phe 1.00 (1.00); His 0.96 (1.00); Arg 1.02 (1.00). ~~The~~ Figure 8 displays the EPR spectra of 0.25 mM Poac^7-AII at pH 3, 6 and 9 aqueous solution. No significant variation on the rotational correlation time values was observed for this paramagnetic AII analogue thus suggesting that its ~~conformations~~ **conformation** is not affected by the pH of the media.

SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELING DIFFERENT MACROMOLECULES AND SYSTEMS

FIELD OF THE INVENTION

This invention relates to the chemical synthesis of a new paramagnetic β-amino acid derivative containing a stable nitroxide radical moiety inserted in its pyrrolidine structure and in which the 9-fluorenylmethyloxycarbonyl group (Fmoc) was chosen as its amine function protecting group. This paramagnetic compound is a new type of spin probe (or spin label) and can be used as alternative report molecule for labeling peptide sequences, other macromolecules and systems where electron paramagnetic resonance spectroscopy (EPR) can be applied. This compound can be used also for other spectroscopic methods such as fluorescence and nuclear magnetic resonance since its paramagnetism may affect the spectra of these methodologies. Due to the presence of both carboxyl and amine groups in its structure, this organic compound may be used for labeling a great variety of other molecules or systems containing reactive functions for these two groups.

BACKGROUND AND SUMMARY OF THE INVENTION

The intermediate for the synthesis of the amino acid derivative of this invention contains the structure 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amine-4-carboxylic acid, henceforth denominated as Poac, synthesized more than two decades ago (see, Tetrahedron 491-499 [1965] and Bull. France, 3, 815-817 [1967]). The Poac derivative containing the Fmoc protecting group (2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-(9-fluorenylmethyloxycarbonyl)-amine-4-carboxylic acid) is the novel spin probe derivative of the present invention, which also includes the synthesis and use in EPR of this spon probe derivative. This new compound allows the insertion of Poac as a usual amino acid at any position of a peptide sequence, and its denomination will be Fmoc-Poac or EPM-5 in this application. The chemical structure of this paramagnetic molecule is represented in Figure 1.

Electron paramagnetic resonance (EPR) [described in *Biological Magnetic Resonance*, Berliner, L. J. and Reuben, J., eds., Plenum Publishing, New York, (1989)], is a modern and very useful spectroscopic method because it allows the study of paramagnetically labeled macromolecules or biological systems regarding their conformation, mobilities, inter- or intra-molecular interactions, structuring states, and the like. The wide spectrum of EPR application is already detailed in the literature (see, *Free Nitroxyl Radical*, Rozantsev, E.G., Ulrich, H., ed., Plenun Press, London, 1970), where a great variety of spin labels, i.e., chemical compounds which are paramagnetic due to the presence of an unpaired electron in their structure, is listed. They are, therefore, a class of free radicals but are necessarily stable under conditions around normal temperature and physiological pH and also allow several chemical reactions or experiments without affecting their free radical moiety.

Amongst the most commonly used spin labels one can pick out the nitroxide group-containing molecules and where the unpaired electron locates. The most significant progress in the EPR field for labeling of relevant biological structures such as peptides and proteins was achieved with this class of spin probes. Almost two decades ago appeared the first EPR application in the solid phase peptide synthesis methodology [(*The Peptides: Analysis, Synthesis and Biology*, vol. 2, Academic Press, New York, (1980)]. This approach was introduced by our group using, instead, another nitroxide-containing spin label, denominated at that time as Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine-4-carboxylic acid), which protected its amine function with the acid labile tert-butyloxycarbonyl (Boc) group [(see, Braz. J. Med. Biol. Res. *14*, 173, (1981) and Biochim. Biophys. Acta, *742*, 63, (1983)]. Thus the Boc-Toac spin probe was the first in the literature used to label a peptide sequence as an amino acid. However, due to chemical particularities of the peptide synthesis methodology, it was only possible to couple the Toac group at the peptide N-terminal position. To overcome this shortcoming, an alternative

strategy was published by us which finally allowed the insertion of the spin label internally to the peptide sequence [see, J. Am Chem. Soc. *115,* 11042 (1993)].

An impressive increase in the application of EPR for peptide chemistry field was further observed in reports investigating peptide conformational properties [v.g. J. Am. Chem. Soc. 117, 10555 (1995); FEBS Lett. *375*, 239 (1995); Biopolymers *42*, 821 (1997)] or of peptidyl-resin solvation (Tetrahedron Lett. *375*, 239 [1995]; Biopolymers *42*, 821 [1997]). As an amino acid, Toac was introduced in different positions of some biologically active peptides such as angiotensin II and bradykinin, but a partial or total loss of their biological potencies were observed due to the introduction of a non natural compound in their structures. [*Peptides 1996*, R. Ramage and R. Epton, eds., Mayflower Scientific Co. p. 673 (1998)].

However, we recently described the synthesis of a peptide hormone labeled with Toac where its biological potency was entirely preserved. [e.g., FEBS Lett. *446*, 45 (1999)]. This result was obtained with the tridecapeptide α-melanocyte stimulating hormone analogue, owing to its potentialities in a great number of chemical-biological assays (this analogue is paramagnetic, naturally fluorescent and fully active, and was disclosed in commonly owned U.S. patent application Serial No. 09/935, 760, entitled "Paramagnetic And Active Analogue (EPM-2) Of Melanocyte Stimulating Hormone Containing Amino Acid-Type Stable Free Radical").

In spite of these results, one still remaining shortcoming in the use of Toac in peptide chemistry is the severe difficulty in coupling the subsequent amino acid residue of the peptide sequence during the synthesis. This difficulty seems to be due to the low nucleophilicity of the Toac amine group, whose pKa of 8 (when in free state) decreases to about 5.5 when bound to the N-terminal portion of a peptide chain [v.g Braz J. Med. Biol. Res. *14*, 173 (1981) and Biochim. Biophys. Acta. *742*, 63 (1983)]. Several recouplings and an increase in the temperature of the coupling reaction are usually necessary to assure

complete incorporation of the subsequent amino acid of the desired peptide sequence [J. Am. Chem. Soc. *115*, 11042 (1993)].

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a depiction of the structure of Fmoc-Poac or EPM-5 according to this invention.

Figure 2 is a mass spectrum of Poac.

Figure 3 is a high performance liquid chromatography (HPLC) profile of Poac showing a single peak.

Figures 4A and 4B depict EPR spectra of Poac in water and Fmoc-Poac in dimethylformamide, respectively.

Figure 5 is a mass spectrum of Fmoc-Poac.

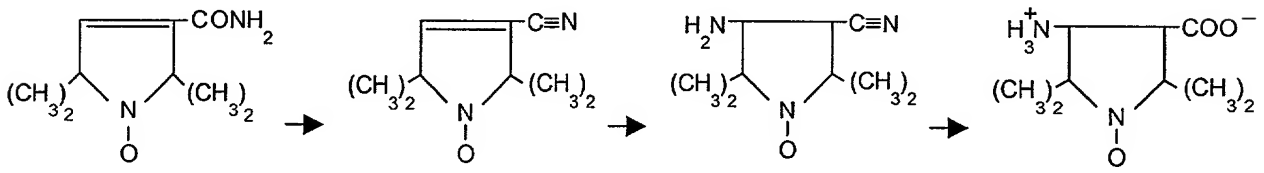
Figure 6 is a HPLC profile of Poac^7 showing a single peak.

Figure 7 is a mass spectrum of Poac^7-angiotensin II.

Figures 8A, 8B and 8C depict EPR spectra of Poac^7-angiotensin II at pH 3.0, 6.0 and 9.0, respectively.

DETAILED DESCRIPTION OF THE INVENTION

In order to overcome the limitations of the Toac probe to find an alternative spin label which may induce differentiated conformational constraints in peptide structures in accordance with this invention, we synthesized Fmoc-Poac according to the synthetic route shown below that is partially described in Tetrahedron, 491-499 (1965); Bull. Soc..Chim. France, *3*, 815 (1967):



P1 **P2** **P3** **P4**

Step 1 - Synthesis of 2,2,5,5-tetramethylpyrroline-1-oxyl-3-cyano (P2)

This product was synthesized by treating compound P1 (from Sigma Co) with tosyl (p-toluenesulfonate) chloride in dry pyridine. To 28.7 g (1.5×10^{-1} mol) of tosyl chloride, 15.3 g (8.35×10^{-2} mol) of P1 dissolved in 100 mL of dry pyridine was added and left at room temperature for 48 hours. After this period, 10 g of KOH dissolved in 250 mL of water were added, and the mixture was heated up to 80°C. After cooling, the product was extracted with sulfuric ether, washed with diluted HCl, diluted $NaHCO_3$ solution, water and dried over anhydrous Na_2SO_4. After evaporation of the solvent under reduced pressure 12.76 g (yield= 92%) of an orange powder was obtained and further purified in an alumina column using benzene as an eluent. The product (P2) showed a single spot in thin layer chromatography with following characteristics: M.P.= 62-63°C, M+ = 165; Elementary analysis found: C, 65.36% , H, 7.50% e N, 17.10 % ; calculated : C, 65.43% , H, 7.93% e N, 16.96%).

Step 2 : Synthesis of 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amine-4-cyano (P3).

In a 3 L round bottom flask, 600 mL of liquid ammonia, 9 g (5.44×10^{-2} mol) of P2 and 120 mL of water were added. The flask was tightly closed to maintain the mixture under pressure and left at room temperature. After 3 days, the ammonia was eliminated and the product extracted with chloroform. The crude product crystallized from ether-petroleum ether yielded 9.37 g (yield = 94%) of a yellow powder with the following characteristics: M+= 182; P.F. 84-85 °C; Elementary analysis found: C 58.98%; H, 8.37%; N, 22.21%; calculated ($C_9H_{16}ON_3$): C, 59. 31%; H, 8.85 % ; N, 23.06 %).

Step 3 - Synthesis of 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amino-4-carboxylic-acid (P4) (Poac).

8 g (4.38×10^{-2} mol) of P3 and 40 g of $Ba(OH)_2$ were suspended in 600 mL of water and added to a 3 L round bottom flask. The flask was tightly closed and heated up to 120°C for 90 minutes. After cooling, the mixture was neutralized with an excess of dry ice

and filtered. The aqueous solution was concentrated under reduced pressure and yielded 8 g of crude product (yield: 90%) that was crystallized from 90% ethanol. The product presented the following characteristics: M.P. = 212°C (melts with sublimation); M+ = 201 (Figure 2), single peak in HPLC (Figure 3); elementary analysis found : C, 53.1% ; H, 8.28 %; N, 13.95 % ; calculated ($C_9H_{17}N_2O_3$): C, 53.71 % ; H, 8.52; N, 13.92 Infra-red (KBr) : cm^{-1} : 3084, 2872, 2792, 2548 and 2132 (NH+3); 1643 ν_{AS} (NH+3) ; 1574 (ν_{AS} , C=O); 1456 (δ CH3); 1396 and 1376 (gem-dimethyl and COO-); 782 (δ C=O). Figure 4A shows the EPR spectrum of POAC in aqueous solution, pH 5. The calculated values for the two rotational correlation times (τ_B and τ_c) were 0.509 x 10^{-10} and 0.597 x 10^{-10}, respectively.

Preparation of Fmoc-Poac

201 mg (1 mmol) of P4 was dissolved in 1.5 mL of water in presence of 286 mg of sodium carbonate.$10H_2O$ in which 337 mg (1 mmol) of Fmoc-succinimidyl-carbonate dissolved in 1.5 mL of acetone was added drop-wise. The reaction was carried out at room temperature with stirring, and the pH was maintained around 9 by addition of sodium carbonate. After 3 hours, the mixture was diluted with 25 mL of water and acidified with 1 N HCl until it reached pH 2. The desired product was extracted with ethyl acetate, washed with small portions of water, dried over anhydrous sodium sulfate. After filtration, the solvent was evaporated under reduced pressure. The crude product was crystallized twice with chloroform and yielded 380 mg (yield : 90%). The product was characterized by mass and infra-red spectroscopy, elementary analysis and EPR. Characteristics: M+ = 423 (Figure 5); elementary analysis found: C, 67.9 % ; H, 6.35 %; N, 6.60 ; calculated ($C_{24}H_{27}O_5N_2$): C, 68. 08% ; H, 6.28 % ; N, 6.62%; IR (KBr) cm^{-1}: 3444-3338 (broad band OH and -CONHR); ~3030 (νAr CH); 3000-2700 (νAr COOH); 1723 (R-O-C-ON- and COOH); 1543 (δ_{NH} and νCN); 1450 (δ CH_3); 1235-1150 (gem-dimethyl group). The EPR spectra of Fmoc-Poac in dimethylformamide is represented in Figure 4B and the calculated τ_B and τ_c values are 1.14 x 10^{-10} s.rad^{-1} and 1.79 x 10^{-10} s. rad^{-1}, respectively.

Synthesis of Poac^7-angiotensin II

Angiotensin II analogue labeled with the spin probe Poac (Asp-Arg-Val-Tyr-Ile-His-Poac-Phe) was synthesized in 0.15 mmol scale, by the solid phase method already mentioned and with alteration to provide the insertion of this marker in the middle of the peptide chain. [J. Am. Chem. Soc. *115*, 11042 (1993)]. Fmoc-Phe-Wang-resin [J. Am. Chem. Soc. *95*, 1328 (1973)] with 0.41 mmol/g substitution degree acquired commercially was used. All couplings were carried out using 2.5 fold excess for the Fmoc-amino acids and 3 fold excess for Fmoc-Poac. The acylating reagents for coupling were diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole in a dichloromethane:dimethylformamide mixture (1:1, v/v) as a solvent. The Fmoc deprotection was performed with 20% piperidine in dimethylformamide (v/v) for 20 min.

Interestingly, this synthesis demonstrated that the pyrrolidine structure of the Poac spin label allowed much easier incorporation of the subsequent amino acid residue than has been observed during the Toac^7-AII synthesis. In this latter case, repetitive recoupling reactions were necessary, including an increase in temperature. These procedures were not necessary in the case of the Poac derivatives, thus demonstrating that the Poac amine group reactivity is much higher than that of Toac.

After the completion of the synthesis, the peptide was cleaved from the resin in anhydrous HF containing 10% of a p-cresol and dimethylsulfide mixture, at 0°C for 90 min. The crude peptide obtained after extraction and lyophilization (125 mg) was dissolved in 70 mL of water and the pH was raised to 10 with ammonium hydroxide and stirred for 2 h, to reverse the nitroxide protonation that occurs during the HF treatment. After lyophilization, the peptide was purified by preparative HPLC (high performance liquid chromatography) using a reverse phase C_{18} (25 x 250 mm) and ammonium acetate 0.02 M, pH 5 and acetonitrile 60% in water, as solvents A and B, respectively. The linear gradient applied was from 20-65% of B for 135 min.

The homogeneity of the peptide was confirmed through analytical HPLC (Figure 6), mass spectrometry, M+ = 1132.55 (Figure 7), and the amino acids analysis showed the expected composition : Asp 0.95 (1.00); Val 0.96 (1.00); Ile 1.20 (1.00); Tyr 1.02 (1.00); Phe 1.00 (1.00); His 0.96 (1.00); Arg 1.02 (1.00). Figure 8 displays the EPR spectra of 0.25 mM Poac^{7}-AII at pH 3, 6 and 9 aqueous solution. No significant variation on the rotational correlation time values was observed for this paramagnetic AII analogue thus suggesting that its conformation is not affected by the pH of the media.

SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELLING CHEMICAL AND BIOLOGICAL MACROMOLECULES

This invention refers to the chemical synthesis of a new paramagnetic β-amino acid derivative containing the stable nitroxide radical moiety inserted in its pyrrolidine structure and the 9-fluorenylmethyloxycarbonyl group (Fmoc) was chosen as its amine function protecting group. This paramagnetic compound is therefore a new type of spin probe (or spin label) and it can be used as alternative report molecule for labeling peptide sequences, other macromolecules and systems where the electron paramagnetic resonance spectroscopy (EPR) can be applied. The use of this compound can be extended also for other spectroscopic methods such as fluorescence and nuclear magnetic resonance since its paramagnetism may affect the spectra of this methodologies. Due to the presence of both carboxyl and amine groups in its structure, the use of this organic compound may be extended for labeling a great variety of other molecules or systems containing reactive function for these two groups.

The intermediate for the synthesis of the amino acid derivative of the present patent contains the structure 2,2,5,5-tetramethylpyrrolidine-1-oxil-3-amine-4-carboxylic acid henceforth denominated as Poac, synthesized more than two decades ago (v.g. Tetrahedron 491-499 [1965] and Bull. France, 3, 815-817 [1967]). Thus, the Poac derivative containing the Fmoc protecting group (2,2,5,5-tetramethylpyrrolidine-1-oxil-3-(9-fluorenylmethyloxicarbonyl)-amine-4-carboxylic acid) is the novel spin probe derivative of the present patent of invention. This new compound allows the POAC insertion as an usual amino acid at any position of the peptide sequence and its denomination will be

Fmoc-Poac or EPM-5 in this descriptive report. The chemical structure of this paramagnetic molecule is represented in **Figure 1**.

The electron paramagnetic resonance (EPR) [*in* Biological Magnetic Resonance, Berliner, L. J. and Reuben, J., eds, Plenum Publishing, New York, (1989)], is a modern and very useful spectroscopic method because it allows studies of any paramagnetically labeled macromolecules or biological systems regarding their conformation, mobilities, inter - or intra-molecular interactions, structuring state, etc. The wide spectrum of EPR application is already detailed in the literature (v.g. Free Nitroxyl Radical, Rozantsev, E.G., Ulrich, H., ed., Plenun Press, London, 1970), where a great variety of spin labels, i.e., chemical compounds which are paramagnetic due to the presence of an unpaired electron in its structure, is listed. They are therefore a class of free radical but must be necessarily stable towards normal temperature and physiological pH and also allow several chemical reactions or experiments without affecting its free radical moiety.

Amongst the most commonly used spin labels one can detach the nitroxide group-containing molecules and where the unpaired electron locates. The most significant progress in the RPE field for labeling of relevant biological structures such as peptides and proteins was achieved with this class of spin probes. Almost two decades ago appeared the first RPE application in the solid phase peptide synthesis methodology [(The Peptides: Analysis, Synthesis and Biology, vol. 2, Academic Press, New York, (1980)]. This approach was introduced by our group using instead, an other nitroxide-containing spin label denominated at that time as **Toac** (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine-4-carboxylic acid protected at it amine function with the acid labile tert-butiloxicarbonyl (Boc) group [(v.g. Braz. J. Med. Biol. Res. *14*, 173, (1981) and Biochim. Biophys. Acta, *742*, 63,

(1983)]. Thus the Boc-Toac spin probe was the first in the literature used to label a peptide sequence as an amino acid. However, due to chemical particularities of the peptide synthesis methodology, it was only possible to couple the Toac group at the peptide N-terminal position. To overcome this shortcoming, an alternative strategy was published by us which finally allowed the insertion of the spin label internally to the peptide sequence [v.g. J. Am Chem. Soc.*115*, 11042 (1993)].

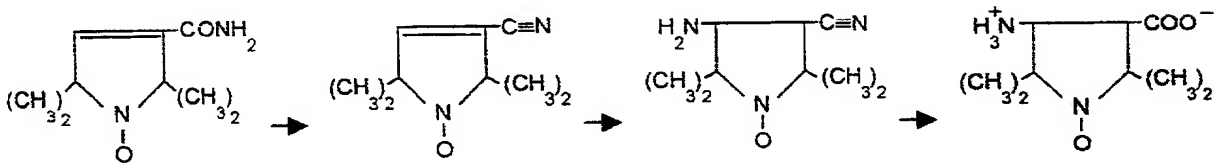
An impressive increase in the application of RPE for peptide chemistry field was further observed with reports investigating peptide conformational properties [v.g. J. Am. Chem. Soc. 117, 10555 (1995); FEBS Lett. *375*, 239 (1995); Biopolymers *42*, 821 (1997)] or of peptidil-resin solvation (Tetrahedron Lett. *375*, 239 [1995]; Biopolymers *42*, 821 [1997]). As an amino acid, TOAC was introduced in different positions of some biologically active peptides such as angiotensin II and bradykinin but a partial or integral loss of their biological potencies were observed due to the introduction of a non natural compound in their structures. [Peptides 1996, R. Ramage and R. Epton, eds, Mayflower Scientific Co. p. 673, (1998)].

However, we recently described the synthesis of a peptide hormone labeled with Toac and where its biological potency was entirely preserved. [v.g FEBS Lett. *446*, 45 (1999)]. This result was obtained with the tridecapeptide α-melanocyte stimulating hormone analogue and owing to its potentialities in a great number of chemical-biological assays (this analogue is paramagnetic, naturally fluorescent and fully active), was submitted for the patent process (PI 9900595, February 24, 1999 : "Synthesis of the first paramagnetic and active α-melanocyte stimulating hormone analogue containing free radical amino acid".

In spite of these results, one still remaining shortcoming to the use of Toac in peptide chemistry refers to the severe difficulty in coupling the subsequent amino acid residue of the peptide sequence during the synthesis. It seems to

be due to the low nucleophilicity of Toac amine group whose pKa of 8 (when in free state) decreases to about 5.5 when bound to the N-terminal portion of a peptide chain [v.g Braz J. Med. Biol. Res. *14*, 173 (1981) and Biochim. Biophys. Acta. *742*, 63 (1983)]. Several recouplings and the increase on the temperature of the coupling reaction are usually necessary to assure complete incorporation of the subsequent amino acid of the desired peptide sequence [J. Am. Chem. Soc.*115*, 11042 (1993)].

Aiming to overcome this limitation inherent to the Toac probe and searching for an alternative spin label which may induce a differentiated conformational constraints in peptide structures, we decided to synthesize the **FMOC-POAC** according to partially described synthetic route shown below [Tetrahedron, 491-499 (1965); Bull. Soc..Chim. France, *3*, 815 (1967)] :



P1 **P2** **P3** **P4**

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This product was synthesized by treating the compound P1 (from Sigma Co) with tosil-chloride in dry pyridine. To 28.7 g (1.5×10^{-1} mol) of tosil-chloride, 15.3 g (8.35×10^{-2} mol) of P1 dissolved in 100 mL of dry pyridine was added and left at room temperature for 48 hours. After this period, 10 g of KOH dissolved in 250 mL of water were added and the mixture was heated up for 80°C. After cooling, the product was extracted with sulfuric ether, washed with diluted HCl, diluted $NaHCO_3$ solution, water and dried over anhydrous Na_2SO_4. After evaporation of the solvent under reduced pressure 12.76 g (yield = 92%) of an orange powder was obtained and further purified in an

alumina column using benzene as eluent. The product (P2) showed a single spot in thin layer chromatography and with following characteristics: M.P. = 62-63°C, M+ = 165; Elementary analysis: found: C, 65.36% , H, 7.50% e N, 17.10 % ; calculated : C, 65.43% , H, 7.93% e N, 16.96%).

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8 g (4.38×10^{-2} mol) of P3 and 40 g of $Ba(OH)_2$ were suspended in 600 mL of water and added to a 3 L round bottom flask. The flask was tightly closed and heated up to 120°C for 90 minutes. After cooling, the mixture was neutralized with excess of dry ice and filtered. The aqueous solution was concentrated under reduced pressure and yielded 8 g of crude product (yield: 90%) that was crystallized from 90% ethanol. The product presented the following characteristics: M.P. = 212°C (melts with sublimation); M+ = 201 (**Figure 2**), single pick in HPLC, (**Figure 3**); elementary analysis : found : C, 53.1% ; H, 8.28 %; N, 13.95 % ; calculated ($C_9H_{17}N_2O_3$): C, 53.71 % ; H, 8.52; N, 13.92 Infra-red (KBr) : cm^{-1} : 3084, 2872, 2792, 2548 and 2132 (NH+3); 1643 ν_{AS} (NH+3) ; 1574 (ν_{AS} , C=O); 1456 (δ CH3); 1396 and

1376 (gem-dimethyl and COO-); 782 (δ C=O). The **Figure 4A** shows the EPR spectrum of POAC in aqueous solution, pH 5. The calculated values for the two rotational correlation times (τ_B and τ_c) were 0.509 x 10^{-10} and 0.597 x 10^{-10},respectively.

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method already mentioned and with alteration to provide the insertion of this marker in the middle of the peptide chain. [J. Am. Chem. Soc. *115*, 11042 (1993)]. FMOC-Phe-Wang-resin [J. Am. Chem. Soc. *95*, 1328 (1973)] with 0.41 mmol/g substitution degree acquired commercially was used. All couplings were carried out using 2.5 fold excess for the FMOC-amino acids and 3 fold excess for Fmoc-POAC. The acylating reagents for coupling were diisopropylcarbodiimide (DIC) and 1-hidroxibenzotriazole in dichloromethane : dimethylformamide mixture (1:1,v/v) as solvent. The Fmoc deprotection was performed with 20% piperidine in dimethylformamide (v/v) for 20min.

Interestingly, this synthesis demonstrated that the pyrrolidine structure of the Poac spin label allowed much easier incorporation of the subsequent amino acid residue than the observed during the Toac^7-All synthesis. In this latter case, repetitive recoupling reactions were necessary including the increase in the temperature. These procedures were not necessary in the case of Poac derivative thus demonstrating that the Poac amine group reactivity is much higher than that of Toac.

After the completion of the synthesis, the peptide was cleaved from the resin in anhydrous HF containing 10% of the p-cresol and dimethylsulfide mixture, at 0°C for 90 min. The crude peptide obtained after extraction and lyophilization (125 mg) was dissolved in 70 mL of water and the pH was raised to 10 with ammonium hydroxide and stirred for 2 h, to revert the nitroxide protonation that occurs during the HF treatment. After lyophilization, the peptide was purified by preparative HPLC (high performance liquid chromatography) using a reverse phase C_{18} (25 x 250 mm) and ammonium acetate 0.02 M, pH 5 and acetonitrile 60% in water, as solvents A and B, respectively. The linear gradient applied was from 20-65% of B in 135 min.

The homogeneity of the peptide was confirmed through analytical HPLC (**Figure 6**), mass spectrometry, M+ = 1132.55 (**Figure 7**) and the amino acids analysis showed the expected composition : Asp 0.95 (1.00); Val 0.96 (1.00); Ile 1.20 (1.00); Tyr 1.02 (1.00); Phe 1.00 (1.00); His 0.96 (1.00); Arg 1.02 (1.00). The **Figure 8** displays the EPR spectra of 0.25 mM $Poac^7$-All at pH 3, 6 and 9 aqueous solution. No significant variation on the rotational correlation time values was observed for this paramagnetic All analogue thus suggesting that its conformations is not affected by the pH of the media.

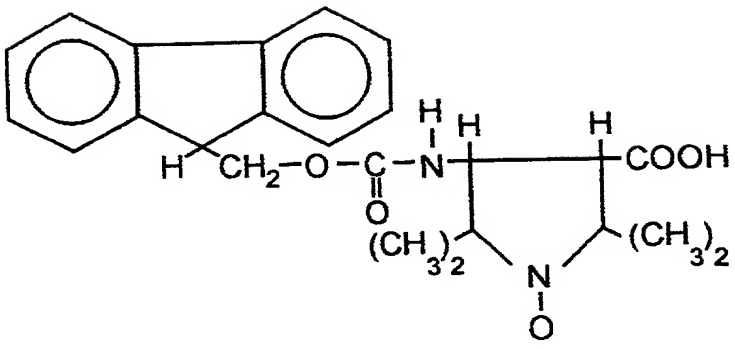
CLAIMS

1º)."**SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELING DIFFERENT MACROMOLECULES AND SYSTEMS OF CHEMICAL-BIOLOGICAL INTEREST**", characterized by synthesizing the novel paramagnetic β-amino acid-type derivative 2,2,5,5-tetramethylpyrrolidine-N-oxyl-(9-fluorenylmethyloxycarbonyl)-3-amine-4-carboxylic acid that was synthesized from the following sequential intermediates : (a) 2,2,5,5-tetramethylpyrroline-1-oxyl-3-cyano; (b) 2,2,5,5-tetramethylpyrrolidine-N-oxyl-3-amino-4-cyano and (c) 2,2,5,5-tetramethylpyrrolidine-3-amino-4-carboxilic acid (POAC), yielding the derivative above mentioned denominated Fmoc-POAC or EPM-5.

2º) **"SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELING DIFFERENT MACROMOLECULES AND SYSTEMS OF CHEMICAL-BIOLOGICAL INTEREST"**, according to the precedent claim 1 and characterized by the fact that Fmoc-POAC can be coupled to macromolecules or systems through its carboxyl function, irrespective of its use or not in further electron spin resonance method.

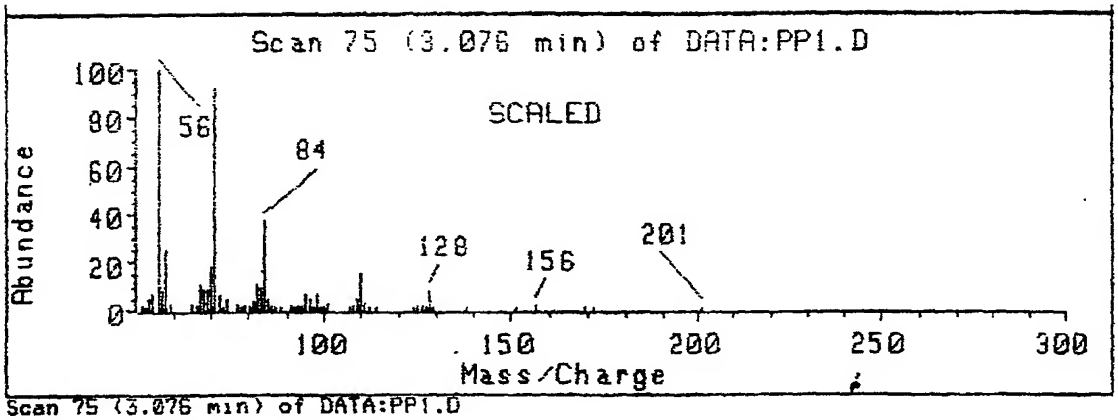
3º) **"SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELING DIFFERENT MACROMOLECULES AND SYSTEMS OF CHEMICAL-BIOLOGICAL INTEREST"**, according to the previous claims and after its incorporation to a molecule or a structure, the Fmoc protecting group of the POAC compound can be removed for further coupling of different chemical derivatives at its free amine function.

Figure 1



Structure of Fmoc-Poac.

Figure 2



Mass spectrum of POAC.

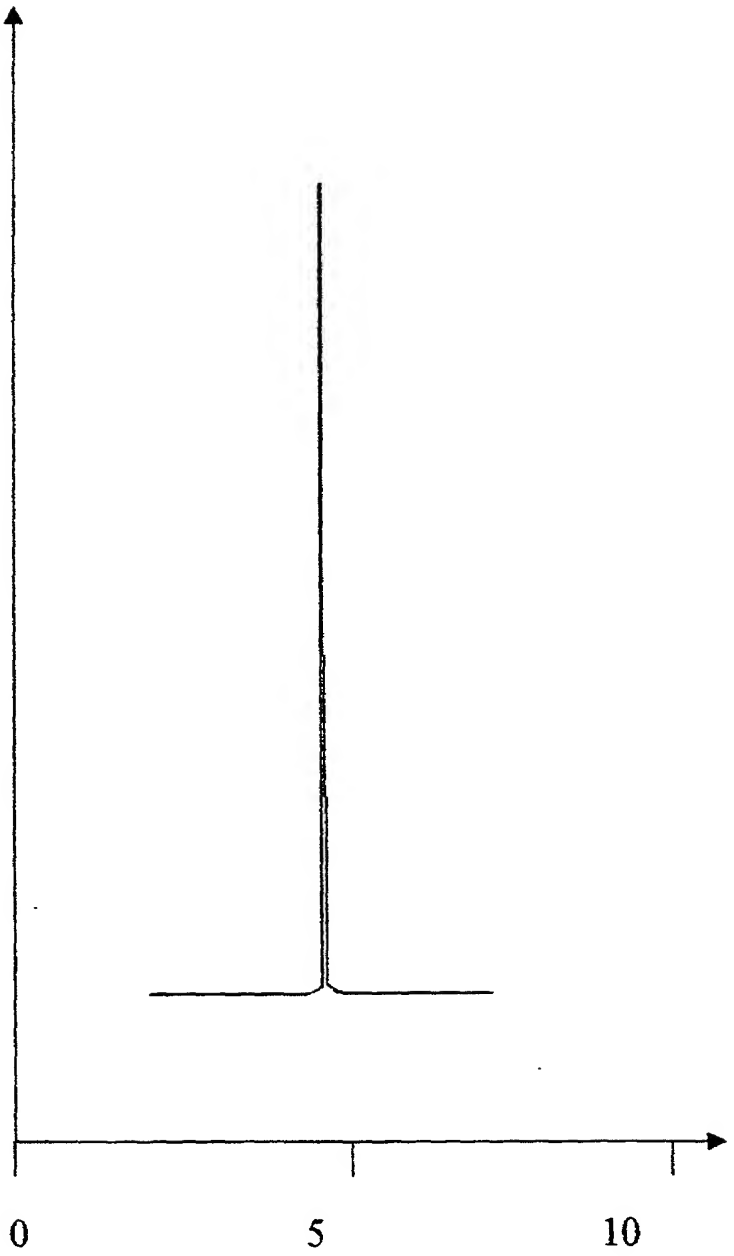
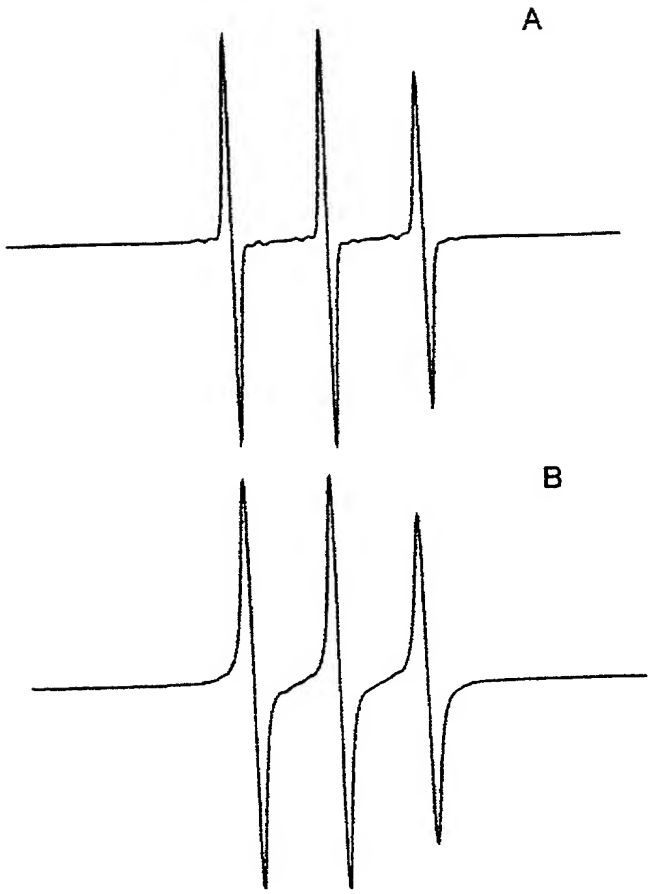


Figure 3

HPLC profile of 2,2,5,5-tetramethyilpyrrolidine-1-oxil-3-amine-4-carboxylic acid (Poac). Experimental conditions: C_8 (4,6 x 150 mm) column. Eluent A: NaH_2PO_4 0,1M/water and, B: acetonitrile 90%/water ; 1-21% of B in 10 min.

Figure 4



RPE spectra of 1×10^{-4} M (A) Poac in water, pH 5; (B) Fmoc-Poac in dimethylformamide.

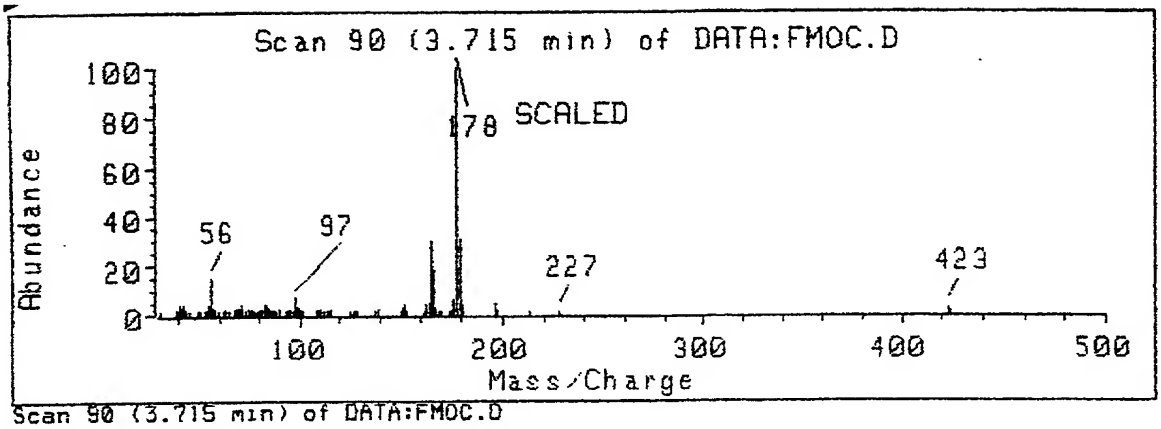


Figure 5: Mass spectrum of Fmoc-POAC.

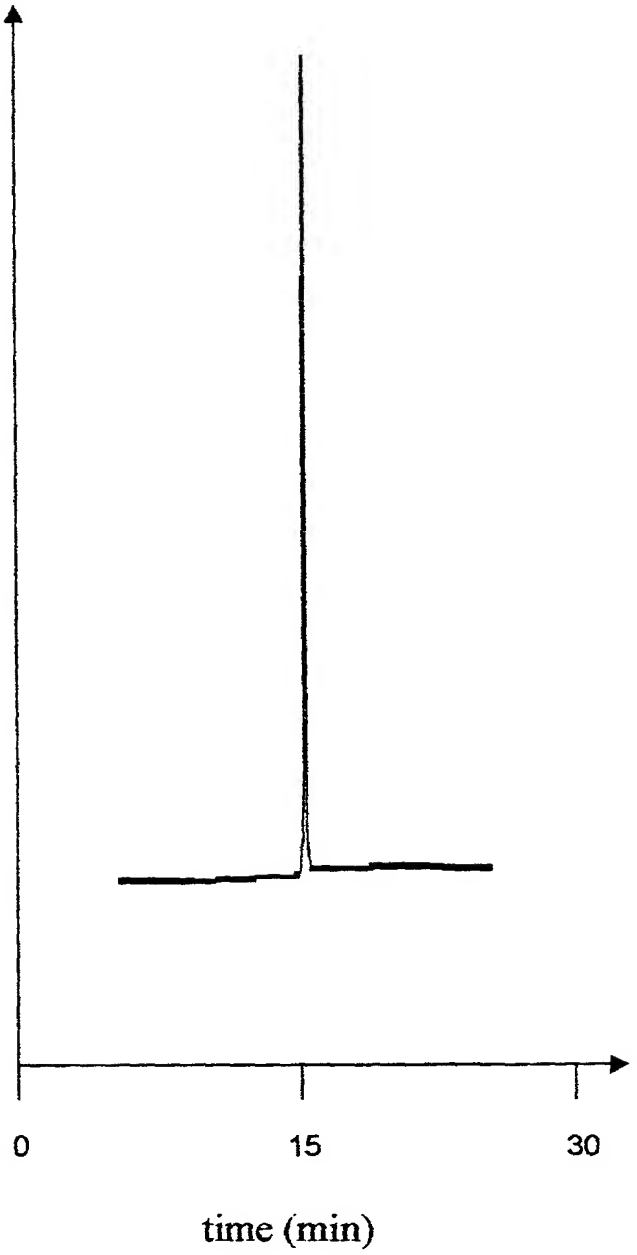


Figure 6

HPLC profile of Poac[7] - AII. Column RF C_{18} (4,6 x 150 mm). Eluent A: 0,1% aqueous TFA and eluent B, 0,1% TFA in 60% aqueous acetonitrile. Flow rate, 1.5mL/min. Elution was performed by a linear gradient of 5% to 95% eluent B.

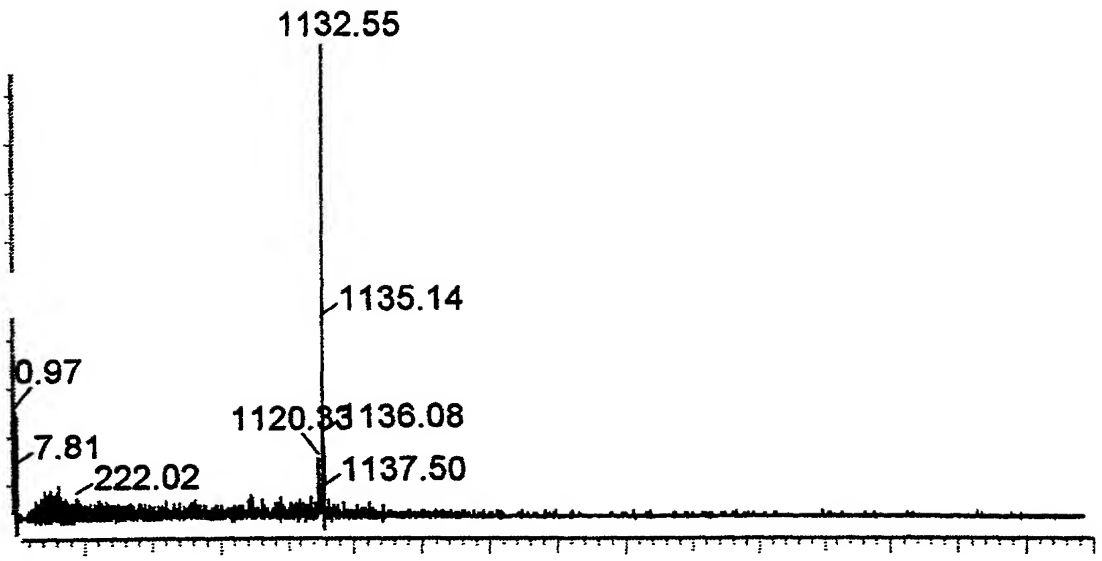


Figure 7

Mass spectrum of Poac^{7}-Angiotensin II.

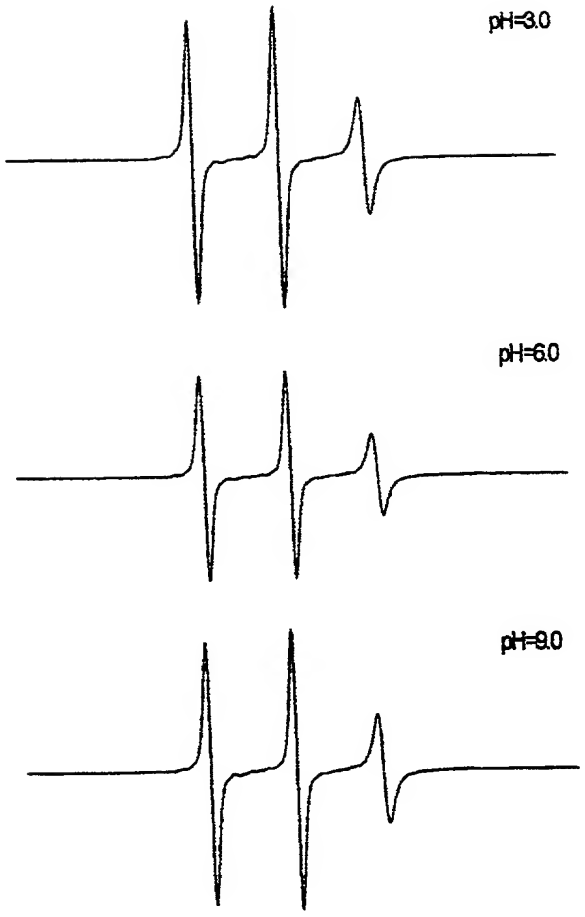


Figure 8

RPE spectra of 2.5×10^{-4} M Poac^{7}-AII in pH 3, 6 and 9.

PATENT
Docket No. 491332000300

DECLARATION FOR UTILITY PATENT APPLICATION

AS A BELOW-NAMED INVENTOR, I HEREBY DECLARE THAT:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: PARAMAGNETIC AND ACTIVE ANALOGUE (EMP-2) OF MELANOCYTE STIMULATING HORMONE CONTAINING AMINO ACID-TYPE STABLE FREE RADICAL, the specification of which is attached hereto unless the following box is checked:

☒ was filed on June 22, 1999, as International Application No. PCT/BR99/00068.

I HEREBY STATE THAT I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE.

I acknowledge the duty to disclose information which is material to the patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

Application No.	Country	Date of Filing (day/month/year)	Priority Claimed?
PI 9903137-0	Brazil	6/22/1999	Yes

I hereby claim benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Serial No.	Filing Date

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status
		☐Patented ☐Pending ☐Abandoned

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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